Journal of Surgical Oncology 64:280–288 (1997)

# Intratumoral Heterogeneity in Primary Breast Carcinoma: Study of Concurrent Parameters

LESLIE G. DODD, MD,[1]* B.-J. KERNS, HT, ASCP,[2] RICHARD K. DODGE,[3] AND LESTER J. LAYFIELD, MD[1]

[1]*Department of Pathology, Duke University Medical Center, Durham, North Carolina*
[2]*Cell Imaging Laboratory, Duke University Medical Center, Durham, North Carolina*
[3]*Comprehensive Cancer Center, Department of Biostatistics, Duke University Medical Center, Durham, North Carolina*

**Background and Objective:** Intratumoral heterogeneity for prognostic factors (ploidy, proliferation, hormone receptor positivity) has been demonstrated in primary breast carcinoma by both flow cytometric and image analysis methods. Previously, heterogeneity in tumors had been demonstrated for only singular parameters. Our objective, using maps of tumors in which discrete regions can be analyzed simultaneously for DNA index (DI) and proliferative activity, was to demonstrate heterogeneity with respect to two parameters and to determine whether any interparametric relationships existed.

**Methods:** We analyzed 25 cases of archived, paraffin-embedded breast carcinoma (ductal) for Feulgen stain DNA analysis and MIB-1 immunohistochemistry using the CAS 200 Image Cytometer. For each tumor, four discrete regions were analyzed including tumor–host tissue interface sectors.

**Results:** Of 25 cases, 19 (76%) were homogeneously diploid or near-diploid aneuploid, and 6 (24%) were heterogeneous. Within the heterogeneous group, all cases had at least one diploid and one or more aneuploid populations from separate discrete regions. Five of six DI heterogeneous tumors displayed diploid values for the overall measurements of the respective tumors, based on analysis of 200 or more nuclei. Eight of 25 cases (32%) showed significant measurable variation for MIB-1 proliferative activity in various sectors of tumor. All the MIB-1 heterogeneous tumors, with one exception, were homogeneously diploid.

**Conclusions:** These findings demonstrate that (1) heterogeneity is present with respect to DI and proliferative activity in breast carcinoma and is relatively common, (2) tumors homogeneous for one parameter may be heterogeneous for another, and (3) heterogeneity for proliferative activity is more common in homogeneously diploid tumors than in heterogeneous/aneuploid tumors. *J. Surg. Oncol. 64:280–288, 1997* 

KEY WORDS: **breast carcinoma; tumor heterogeneity; DNA ploidy; proliferation**

Contract grant sponsor: Duke University Comprehensive Cancer Center
*Correspondence to: Dr. Leslie G. Dodd, Box 3712, Department of Pathology, Duke University Medical Center, Durham, NC 27710
Accepted 6 January 1997

## INTRODUCTION

Assessment of tumor prognosis and diagnosis is no longer restricted to histopathologic and clinical estimates of stage and grade. Increasing emphasis is being placed on measurable, quantitative, or semiquantitative indices, such as DNA content, proliferative activity, hormone receptor status, and oncogene expression. With the increased familiarity with and utilization of these prognostic markers, it has become apparent that neoplasms subject to measurements will often show regional variation in values. This phenomenon, known as intratumoral heterogeneity, has been particularly well documented with respect to DNA content and has been observed in a number of human neoplasms, including renal, ovarian, cervical, prostate, lung, gastroesophageal, colorectal, biliary and hepatocellular, thyroid, oral and laryngeal carcinomas, as well as in sarcomas and gliomas. In breast carcinoma, heterogeneity has been thoroughly documented with respect to DNA content [1–8] and to a lesser extent for hormone receptors [6,9,10] and proliferation indices [6,11].

The significance of heterogeneity in breast carcinoma lies in the extent to which quantitative prognostic factors have become used in the diagnosis and treatment of these tumors. In breast cancer, the significance of ploidy had been demonstrated in many studies; particularly the relationship between aneuploid DNA content and poor prognosis [12–20]. In addition, DNA index (DI) has been demonstrated to correlate with other prognostic variables, including estrogen receptor status [21–23], histologic differentiation [16,24–27], lymph node status [21,22,24], and proliferative index [23,24].

Previously, the study of heterogeneity in breast tumors has been largely confined to DNA content and has been demonstrated for only singular parameters. We are unaware of any demonstrations of relationships of heterogeneity within different quantifiable prognostic variables such as hormone receptor content or proliferative activity. These variables are more difficult to define in terms of heterogeneity because their measurements lack the obvious reference points of euploid and noneuploid that are integral to DNA content analysis.

Our objective in the following was to analyze sections of archived breast carcinoma by image analysis (IA) for marker heterogeneity. The IA method of measurement has an advantage over flow cytometric (FCM) analysis in preservation of tissue architecture as well as allowing visual coordination of the study variable with the tumor morphology. It has also been suggested that IA may be more sensitive than FCM analysis in detecting small aneuploid populations [28,29]. Using IA, we specifically looked for the presence of heterogeneity in breast carcinoma and if tumors homo- or heterogeneous for one parameter or index were likely to be similar for another.

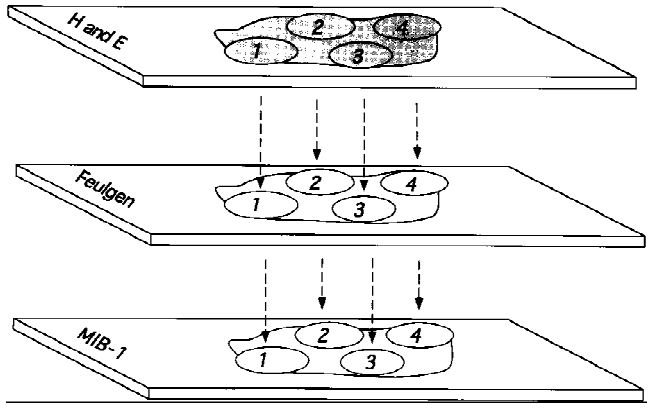


Fig. 1. Schematic representation of preparation of slides for image analysis of DNA and proliferation heterogeneity. Areas selected for analysis were manually transferred from the H&E section to consecutive serial sections of the tumor block stained for DNA analysis (Feulgen stain) and MIB-1 antibody.

## MATERIALS AND METHODS

### Materials

This study was performed on archival material. For inclusion in the study, all tumors selected had to be composed of ductal carcinoma, no lobular carcinomas or prognostically significant variants were analyzed. Tumors were selected that were moderately to poorly differentiated, based on the histologic grading scheme of Bloom and Richardson [30]. These tumors were also exclusively infiltrating with an inconspicuous or absent intraductal component. In order to be divided into four discrete areas appropriate for analysis, sections of tumor had to be relatively large, at least 1.5 cm in diameter.

Twenty five tumors were identified for inclusion in the study. Each histologic section selected for analysis contained a leading edge of tumor (tumor–host tissue interface). For each tumor, four discrete regions were analyzed, three or more of which were peripheral sectors. Four separate, nonoverlapping, or contiguous sectors were selected and marked out on a hematoxylin and eosin (H&E)-stained slide. The areas selected for analysis were then hand transferred to the Feulgen-stained and immunohistochemistry slides, which were prepared from adjacent sections of the tumor block (Fig. 1).

Because the analysis and measurements were performed on an image-based system, all nuclei were visually selected and identified as tumor cells based on malignancy-associated characteristics, including increased nuclear density and irregular chromatin. Likewise, intraductal tumor, benign ductal epithelium and stromal elements were eliminated from the analysis (except to serve as an internal diploid reference). All measurements were performed by a single individual.

Tissue samples were from archives and had been previously formalin-fixed in 10% neutral-buffered formalin and embedded in paraffin. For study, sequential serial

sections of blocks were cut 4–5 μm thick, mounted on slides, and allowed to dry overnight. Deparaffinization was obtained by rinse in three xylene baths and clearing in absolute alcohol.

### DNA: Materials Preparation, Methods, and Definitions

Slides containing tumor for DNA measurement were prepared as 5-μm sections, dried, deparaffinized and rehydrated. The slides were stained by the Feulgen method, using the CAS DNA staining kit (Cell Analysis Systems, Becton Dickinson, San Jose, CA). In brief, slides were placed in 5 N hydrochloric acid for 60 min to hydrolyze the DNA. Then they were stained with the dye solution for 1 hr. The sections were then rinsed in three changes of rinse solution, dehydrated, and coverslipped.

The image cytometer was calibrated by measuring the DNA content of a known standard (tetraploid rat hepatocyte nuclei). Measurement of lymphocytes and stromal cells was used as an internal diploid control. To correct for cut nuclei on tissue sections, the tissue correction factor of the CAS ploidy software was used. This allows for the operator to correct for partial measurements of cut nuclei based on tissue section thickness. More detailed explanation of the correction methodology is given elsewhere [31].

For each sample, the total nuclear optical density of the measured nuclei was obtained at a wavelength of 546 μm. DI was calculated as the DNA content of a neoplastic G0/G1 peak divided by the DNA content of the normal G0/G1 peak. For each tumor, an overall DI was determined for the tumor based on an assessment of 200 nuclei. After this measurement, separate determinations were made for each area based on an analysis of 40–50 nuclei.

A sample was considered diploid ($DI = 1.0 \pm 0.1$) when a single G0/G1 peak was identified, occupying the same position as the diploid control and no other G0/G1 peak with >10% of the total number of nuclei measured was identified. An aneuploid population was identified when one or more G0/G1 peaks outside the diploid range and containing more than 10% of the total number of nuclei measured was present. A ''near diploid, aneuploid'' category was established for values of $1.1 < DI < 1.3$. Unequivocal aneuploid values consisted of $1.3 < DI < 1.7$.

For DI, a tumor was classified as diploid if all sectors were diploid; near-diploid–aneuploid if any sector was near diploid and no other sector aneuploid; aneuploid if at least one sector was aneuploid and no sector diploid; and heterogeneous if at least one diploid and one aneuploid sector were present and the ''overall'' value was nonaneuploid. In the instance in which both aneuploid and nonaneuploid sector values were present but the ''overall'' value was aneuploid, these cases were recorded as ''aneuploid.'' Borderline values of the near-diploid–aneuploid category were considered insufficient evidence of tumor heterogeneity when unaccompanied by clear diploid and/or aneuploid sectors. These were excluded from the definition of tumor heterogeneity to partially compensate for some of the statistical variation in values known to be associated with the inclusion of the nuclear correction factor [32].

### Proliferation: Materials, Preparation, Methods, and Definitions

Proliferation index was determined by quantitation of positive immunohistochemical staining for MIB-1 antibody (AMAC, Westbrook, ME). MIB-1 is a murine monoclonal antibody ($IgG_1$) that reacts with the Ki-67 nuclear antigen expressed by proliferation cells.

Deparaffinized slides were treated with citrate buffer, pH 6.0 (Sigma Chemical Co., St. Louis, MO) for antigen retrieval. Slides were microwaved according to the manufacturer's instructions (AMAC), washed in phosphate-buffered saline (PBS), and then placed in 5% normal goat serum for 20 min. Slides were incubated with MIB-1 antibody overnight at 4 degrees C, then washed in PBS. Goat anti-mouse biotylated antibody (BioGenex, San Ramin, CA) was applied followed by rinse and application of peroxidase-conjugated strepavidin label. Slides were developed with enzyme 3,3′ diaminobenzidine (Sigma), rinsed, and counterstained with 1% methyl green (Sigma).

Determination of proliferation index (PI) was performed using a CAS 200 Image Analysis System (Cell Analysis Systems) in combination with the Quantitative Proliferation Index CAS Software Program (Cell Analysis Systems). The system makes determinations of values based on the number of pixels analyzed containing diaminobenzidine deposition. PI is expressed as a percentage of nuclear area staining positively for MIB-1 relative to the total nuclear area. Control sections, stained with normal mouse IgG1, were prepared in conjunction with each case. These were analyzed first to establish background immunostaining thresholds.

Multiple fields of tumor were analyzed at a magnification of ×400. After mapping, each predetermined discrete region was analyzed and a PI value determined based on analysis of 40–50 nuclei. Reproducibility of measurements was determined by making replicate measurements on homogeneous tumor slides and performing a Pearson correlation coefficient statistical test on the results.

Each sector was also assigned a proliferative activity interpretation based on arbitrary cutpoints defined by the laboratory. The following were used to define low, medium, and high proliferative activity sectors: Low if $0.1\% < PI < 3.9\%$, medium if $4.0\% < PI < 9.9\%$, and high if $PI > 10.0\%$. For PI, a tumor was classified as having a low

activity if all sectors were low; medium activity if any sector was medium and no sector high; high activity if at least one sector was high and no sector low; and heterogeneous if at least one low sector and one high sector were present.

### Statistical Methods

To confirm reproducibility of results for measurements without an internal reference standard (MIB-1 by immunohistochemistry), repeat measurements by two evaluators were performed. The paired t-test was used to assess agreement and consistency. The relationship between DNA index and proliferation index was considered jointly for all 100 sectors (25 samples, four sectors each) using a 3 × 3 classification table and the Spearman rank correlation coefficient [33]. The relationship between the two indices for the 25 samples was assessed using a 2 × 2 table, with the cells consisting of the frequency of nonheterogeneous and heterogeneous tumors observed. McNemar's test was used to compare the proportion of heterogeneity expressed by the two parameters [34]. Proportions and exact 95% confidence intervals (CI) were calculated using standard methods.

## RESULTS

### DNA

An overall DNA index (DI) and DI from four separate sectors were determined in 25 cases (100 sectors). Of the total sectors analyzed, 79% (95% CI: 70%, 87%) were classified as diploid values, 11% (6%, 19%) as near-diploid aneuploid, and 10% (5%, 18%) as aneuploid. The numerical values for each of the sectors and the cases are presented in Table I along with an interpretation for each case.

Thirteen cases (52%) showed overall diploid measurements and diploid measurements for each of the separate sectors. An additional six cases (24%) contained one or more sectors with 1.1 < DI < 1.3. These near-diploid aneuploid cases were excluded from the heterogeneous category because of a preference to maintain stringent criteria for heterogeneity. Six cases (24%) displayed heterogeneity as defined in the *Methods* section. Of these, two cases (15 and 19) showed one or more sectors with a diploid or euploid measurement and one or more sectors with an aneuploid measurement but an overall aneuploid measurement. Four cases (1,3,13,18) contained one or more diploid and one or more aneuploid measurements for separate sectors, but registered an overall diploid DI value (Fig. 2).

H&E-stained sections of the heterogeneous tumors were reviewed to determine whether any histologic correlates to the above finding were apparent. Histologic features examined included extent of tubule formation, cellular pleomorphism, nuclear anaplasia and chromatin pattern. No consistent relationship was uncovered between tumor heterogeneity and histopathologic parameters.

**TABLE I. Summary of DNA Indices for Different Sectors of 25 Primary Breast Carcinomas Analyzed for Intratumoral Heterogeneity**

| | DNA index for sectors | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Overall | Interpretation |
| 1 | 0.92 | 1.37 | 0.93 | 0.93 | 0.97 | Heterogeneous |
| 2 | 1.10 | 0.92 | 0.96 | 0.97 | 1.06 | Diploid |
| 3 | 0.98 | 0.98 | 1.42 | 1.41 | 0.94 | Heterogeneous |
| 4 | 0.96 | 0.95 | 1.02 | 0.96 | 1.02 | Diploid |
| 5 | 1.09 | 1.09 | 1.15 | 1.03 | 0.97 | Near-diploid aneuploid |
| 6 | 0.96 | 0.98 | 1.03 | 0.97 | 1.08 | Diploid |
| 7 | 0.93 | 0.93 | 0.94 | 0.94 | 0.96 | Diploid |
| 8 | 0.99 | 0.99 | 0.92 | 0.96 | 0.97 | Diploid |
| 9 | 0.93 | 0.97 | 0.93 | 0.93 | 0.97 | Diploid |
| 10 | 2.08 | 1.21 | 1.13 | 1.46 | 1.47 | Aneuploid |
| 11 | 0.99 | 1.13 | 0.98 | 1.08 | 1.03 | Near-diploid aneuploid |
| 12 | 0.93 | 0.93 | 0.97 | 0.93 | 0.95 | Diploid |
| 13 | 0.76 | 1.30 | 1.18 | 1.05 | 1.08 | Heterogeneous |
| 14 | 1.15 | 0.92 | 0.98 | 1.03 | 1.07 | Near-diploid aneuploid |
| 15 | 1.80 | 1.31 | 0.99 | 0.93 | 1.26 | Heterogeneous |
| 16 | 0.93 | 1.20 | 1.04 | 0.93 | 0.98 | Near-diploid aneuploid |
| 17 | 1.08 | 0.93 | 0.96 | 1.14 | 0.98 | Near-diploid aneuploid |
| 18 | 1.41 | 1.10 | 1.21 | 0.95 | 1.09 | Heterogeneous |
| 19 | 0.95 | 0.99 | 1.68 | 2.02 | 1.66 | Aneuploid |
| 20 | 0.93 | 0.93 | 0.93 | 0.93 | 0.99 | Diploid |
| 21 | 0.96 | 0.96 | 0.99 | 1.01 | 0.99 | Diploid |
| 22 | 0.93 | 0.93 | 0.95 | 0.96 | 0.96 | Diploid |
| 23 | 1.08 | 1.08 | 1.09 | 1.09 | 1.10 | Diploid |
| 24 | 1.01 | 0.97 | 1.10 | 1.08 | 1.04 | Diploid |
| 25 | 0.93 | 1.20 | 0.93 | 0.96 | 1.10 | Near-diploid aneuploid |

### Proliferative Fraction

Proliferative indices (PI), expressed as a percentage of positive nuclear staining per total cells measured, for each of the cases in the study are given in Table II. Seventeen (68%) of the cases studied failed to show significant variability in proliferation as defined in the Methods section of the study. The overall interpretations for these cases, based on adding and averaging all the values generated in each sector, were low to medium PI in all instances. The paired t-test for the MIB-1 measurements made by two evaluators showed no significant difference between them (mean absolute difference = 0.5, $P = 0.21$).

In the eight (32%) remaining cases, significant measurable variation, i.e., heterogeneity, was demonstrable (Fig. 3). In each of these cases, one or more sectors demonstrated a ''high'' measurement of more than 13%. Also, based on the method of calculating the overall value, most of these cases would have been classified as having a high proliferative index. Heterogeneous cases, 1, 2 and 23 would be classified as having a medium or ''moderate'' proliferative activity. Most heterogeneous cases, as well as those of uniformly low or moderate

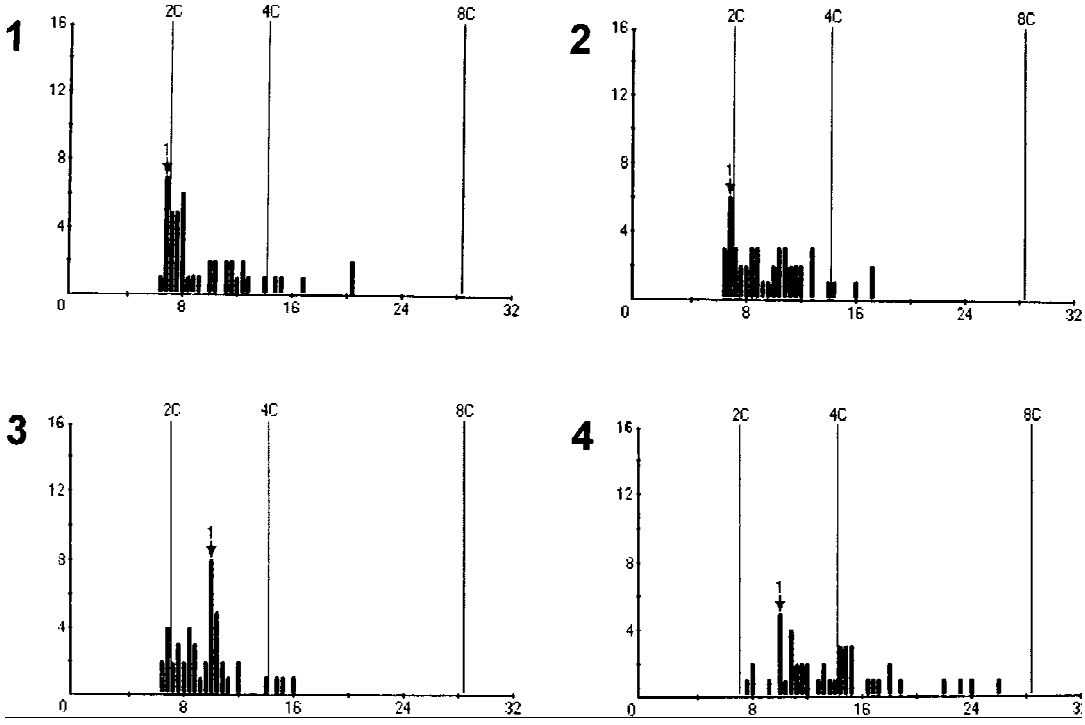


Fig. 2. Composite illustration of DNA histograms from each of four different sectors in a DI heterogeneous tumor. Sectors 1 and 2 are diploid, while 3 and 4 are aneuploid.

proliferative activity, had sections of tumor demonstrating very low or absent proliferative activity (<1%).

Histologic sections from the ''heterogeneous'' neoplasms were examined with an attempt to correlate heterogeneity with a histologic hallmark. No consistent observations were identified. Heterogeneous tumors showed regional variation in numbers of mitoses, but these were few and did not necessarily correspond to the regions identified by MIB-1 as the most proliferative.

### Interparametric Relationships

Tumors identified as heterogeneous in DNA content (samples 1, 3, 13, 15, 18, 19) were, with one exception (sample 1), homogeneously of low to moderate proliferative index. Likewise, tumors heterogeneous in proliferative activity (samples 1, 2, 4, 6, 16, 20, 23, 25) were, with the single exception of sample 1, diploid or near-diploid aneuploid (sample 25). A test using the Spearman rank correlation indicates no significant correlation between DI and PI ($p = 0.28$).

The joint relationship between DI and PI is shown in Table III, where the categories under consideration were nonheterogeneity and heterogeneity. Heterogeneity was observed in 6 [24% (9%, 45%)] cases for DI and in 8 [32% (15%, 54%)] cases for PI. One case [4% (0.1%, 20%)] was heterogeneous simultaneously for both parameters. The remaining five cases that were heteroge-

**TABLE II. Summary of Proliferation Indices Obtained for 25 Primary Breast Carcinomas Analyzed for Intratumoral Heterogeneity**

| | Proliferation (in %) for sector | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | Interpretation |
| 1 | <1 | 2.4 | <1 | 12.4 | Heterogeneous |
| 2 | <1 | 16.3 | 6.8 | <1 | Heterogeneous |
| 3 | 8.2 | <1 | 3.3 | 2.1 | Medium |
| 4 | 16.5 | 3.3 | 6.3 | 28.3 | Heterogeneous |
| 5 | <1 | <1 | 5.6 | <1 | Medium |
| 6 | 18.6 | 12.5 | <1 | <1 | Heterogeneous |
| 7 | <1 | <1 | <1 | <1 | Low |
| 8 | 9.9 | <1 | 3.5 | <1 | Medium |
| 9 | <1 | 1.8 | <1 | 1.3 | Low |
| 10 | <1 | 3.9 | 7.4 | <1 | Medium |
| 11 | 2.2 | 3.0 | 2.6 | 2.6 | Low |
| 12 | <1 | <1 | 1.6 | 1.9 | Low |
| 13 | <1 | 4.5 | <1 | <1 | Medium |
| 14 | 1.3 | 2.8 | <1 | <1 | Low |
| 15 | 4.8 | 1.9 | 2.5 | 4.3 | Medium |
| 16 | 17.3 | 5.6 | 10.1 | 2.6 | Heterogeneous |
| 17 | <1 | 7.4 | <1 | 4.0 | Medium |
| 18 | 5.9 | 8.7 | 9.4 | 7.6 | Medium |
| 19 | 4.5 | <1 | 4.4 | 5.7 | Medium |
| 20 | 11.1 | 2.8 | 11.5 | 33.7 | Heterogeneous |
| 21 | 1.8 | 2.3 | <1 | <1 | Low |
| 22 | <1 | 6.2 | <1 | 3.0 | Medium |
| 23 | 16.9 | <1 | <1 | 4.3 | Hetergeneous |
| 24 | <1 | <1 | 1.2 | 1.8 | Low |
| 25 | <1 | 14.4 | 2.2 | 6.7 | Heterogeneous |

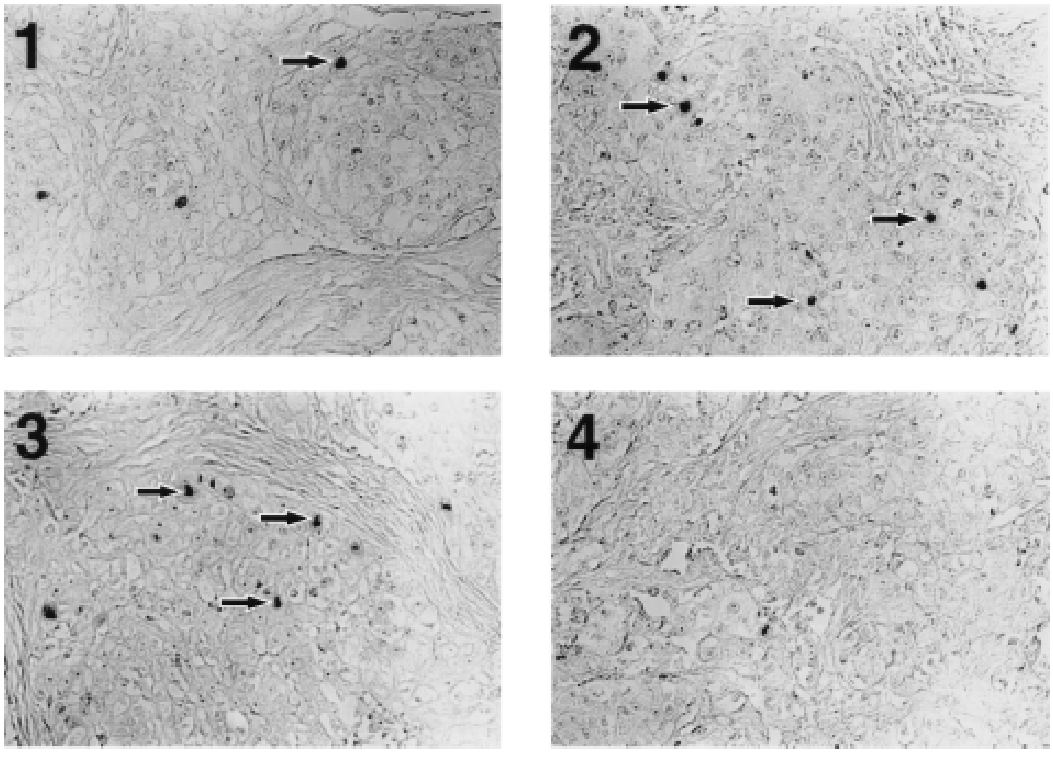


Fig. 3. Composite photomicrographs of different sectors of one tumor demonstrating significant intratumoral heterogeneity with respect to MIB-1 positivity. Proliferation indices are expressed as a percentage of positive nuclear staining (*arrows*). Each positively stained nucleus measures approximately 15 μm in diameter (MIB-1 monoclonal antibody with methyl green counterstain, ×170).

**TABLE III. Breast Carcinoma: Interparametric Relationships Between Tumoral Heterogeneity for DNA Index and Proliferation Index**

| | Proliferation index | | | | | |
|---|---|---|---|---|---|---|
| | Homogeneous | | Heterogeneous | | Total | |
| DNA Index (DI) | n | % | n | % | n | % |
| Homogeneous | 12 | 48 | 7 | 28 | 19 | 76 |
| Heterogeneous | 5 | 20 | 1 | 4 | 6 | 24 |
| Total | 17 | 68 | 8 | 32 | 25 | |

neous for DI were all considered to have medium proliferative activity. The other cases that were heterogeneous for PI were diploid in five and near-diploid in two. McNemar's test [34] indicated that the proportion of heterogeneity was similar for each index ($P = 0.56$).

## DISCUSSION

Although assessment of tumor characteristics such as ploidy, hormone receptor status, and proliferative activity have gained widespread acceptance and utility, it is well recognized that tumors will often fail to behave as predicted based on the information these indices provide. With breast cancer in particular, 30% of patients with diploid tumors and other ''favorable'' prognostic indicators will experience an early relapse and/or shorter survival than predicted by a combination of clinical staging and information from quantitative parameters [35]. Likewise, a negative value for a prognostic variable may be of questionable clinical significance. For example, a percentage of patients with ER-negative tumors will nevertheless respond favorably to adjuvant endocrine therapy [36].

Some of the proposed explanations for the DNA and prognosis discrepancies have included technical [37–39] and subjective errors [40] in the generation and interpretation of quantitative tumor data. Further obscuring the issue of the utilization of DNA indices is the possibility that historical studies on DNA may be partially inaccurate. Recently, the reliability of DNA measurements made from archived, paraffin-embedded materials has been disputed [41–44]. Likewise, the integrity of DNA studies performed with image analysis cytometry on archived, paraffin-embedded material have been questioned [32].

An alternative explanation for the lack of reliability of ancillary information is the existence of small subpopulations of tumor cells reflecting regional variation in a measurable variable. These subpopulations may be clinically significant but missed by conventional measurements. This latter explanation and the concept of intratumoral heterogeneity have gained more widespread acceptance as this phenomenon has been documented with increasing frequency in more human neoplasms. In

breast carcinoma, for example, heterogeneity for DNA content has been demonstrated in 9% (5) to 44% (3) of tumors when specifically studied for heterogeneity; the incidence increasing with tumor size, and number of samples submitted for DNA analysis. Intratumoral heterogeneity has also been used to explain differences in tumor chemosensitivity [45], radiosensitivity [46], and response to hormonal manipulation [47].

The findings from the current study are in agreement with those previously published. In our study, we documented a relatively high incidence of significant heterogeneity (28%) with respect to DNA using stringent standards and omitting the borderline categories of ''near-diploid aneuploid'' and ''peridiploid'' values. Four cases (16% of the study total) demonstrated a potentially significant aneuploid region which was not reflected in the diploid value obtained on conventional measurement of overall DNA content.

Because of lack of easily definable reference points (e.g., diploid, nondiploid, tetraploid), proliferative activity is more difficult to define as heterogeneous. However, practical endpoints, such as those values adopted by the laboratory to separate a ''low'' versus ''high'' proliferative index, provide logical reference points for differences which have potential to be clinically significant. The current study found 32% of tumors to be heterogeneous for the proliferative marker MIB-1 when heterogeneity was defined by the cutpoints of clinically significant laboratory reporting values. This finding has less significance than DNA heterogeneity in that most of the proliferation index ''heterogeneous'' tumors would probably have been identified as having a ''high'' proliferation index. Unlike the circumstances with DNA index, a potentially ''aggressive'' tumor cell population is less likely to be ''missed'' or masked in a sample measurement.

The simultaneous involvement of two variables, in this instance DNA content and proliferative activity, using image analysis techniques, has revealed other characteristics of heterogeneity, the significance of which are unknown. In this study, we showed that heterogeneity is not uniform. Homogeneity for one parameter does not imply homogeneity for another. In fact, in this study 24% and 32% of the samples respectively were heterogeneous for one or the other of the parameters when measured individually and independently.

The current study revealed that paradoxically, a tumor that demonstrated significant heterogeneity for one parameter was likely to be homogeneous for another. With the exception of one case which showed heterogeneity for both variables, all proliferative activity heterogeneous cases were euploid. Likewise, the DNA index heterogeneous tumors were uniformly low to moderately proliferative or had relatively insignificant variations in MIB-1-determined proliferation index. This seemingly inconsistent finding of heterogeneous proliferative activity in diploid tumors has been noted by others [48,49] investigating regional variation.

The documentation of heterogeneity with respect to single parameters has marked significance from both analytic and clinical standpoints. One of the most practical recommendations resulting from the study of heterogeneity or regional tumor variation is that multiple tissue samples be submitted for analysis of quantitative parameters, regardless of methodology chosen. We concur with previous authors who have made these recommendations [4–6,50].

The significance of the findings regarding heterogeneous interparametric relationships, specifically, their lack of correlation or apparent inverse relationship, remains to be seen. Other investigators, noting an association between high levels of variation in proliferative activity in diploid tumors, have suggested that normal (diploid) DNA tumors should proliferate more actively than aneuploid tumors [49]. This observation warrants further investigation. Any information gained from a more thorough understanding of the prevalence and characteristics of heterogeneity in breast carcinoma has profound implications for patient treatment. Moreover, clarification of the nature and extent of breast carcinoma heterogeneity may help explain the discrepancy between predicted tumor behavior based on quantifiable measurements of DNA indices, proliferation index and others and the clinical outcomes seen in a significant proportion of these patients.

## REFERENCES


1. Kallioniemi OP: Comparison of fresh and paraffin-embedded tissue as starting material for DNA flow cytometry and evaluation of intratumoral heterogeneity. Cytometry 1988;9:164–169.
2. Erhardt K, Auer G: Mammary carcinoma: DNA analysis in areas showing different histological features in the same tumor. Acta Pathol Microbiol Immunol Scand A 1986;94:21–28.
3. Bonsing BA, Beerman H, Kuipers-Dijkshoorn N, et al.: High levels of DNA index heterogeneity in advanced breast carcinoma. Cancer 1993;71:382–391.
4. Prey MU, Meyer JS, Stone KR, McDivitt RW: Heterogeneity of breast carcinomas determined by flow cytometric analysis. J Surg Oncol 1985;29:35–39.
5. Fuhr JE, Frye A, Kattine AA, Van Meterer S: Flow cytometric determination of breast tumor heterogeneity. Cancer 1991;67: 1401–1405.
6. Meyer JS, Wittliff JL: Regional heterogeneity in breast carcinoma: thymidine labeling index, steroid hormone receptors, DNA ploidy. Int J Cancer 1991;47:213–220.
7. Beerman H, Smit VT, Kluin PM, Bonsing BA, et al.: Flow cytometric analysis of DNA stemline heterogeneity in primary and metastatic breast cancer. Cytometry 1991;12:147–154.
8. Symmans WF, Liu J, Knowles DM, Inghirami G: Breast cancer heterogeniety: Evaluation of clonality in primary and metastatic lesions. Hum Pathol 1995;26:210–216.
9. Poulson HS, Jensen J, Hermansen C: Human breast cancer: Heterogeneity of estrogen binding sites. Cancer 1981;48:1791–1793.
10. Castagnetta L, Traina A, DiCarlo A, et al.: Do multiple oestrogen receptor assays give significant additional information for the management of breast cancer. Br J Cancer 1989;59:636–638.
11. Siitonen S, Isola JJ, Rantala IS, Helin HJ: Intratumor variation in

cell proliferation in breast carcinoma as determined by antiproliferating cell nuclear antigen monoclonal antibody and automated image analysis. Am J Clin Pathol 1993;9:226–231.
12. Auer GU, Caspersson TO, Wallgren AS: DNA content and survival in mammary carcinoma. Anal Quant Cytol 1980;2:161–165.
13. Coulson PB, Thornwaite JT, Woolley TW, et al.: Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer survival. Cancer Res 1984;44:4187–4196.
14. Auer G, Eriksson E, Azavedo E, et al.: Prognostic significance of nuclear DNA content in mammary adenocarcinomas in humans. Cancer Res 1984;44:394–396.
15. McGuire WL, Dressler LG: Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. J Natl Cancer Inst 1985;75:405–410.
16. Thorud E, Fossa SD, Vaage S, Kaalhus O, et al.: Primary breast cancer: Flow cytometric DNA pattern in relation to clinical and histopathologic characteristics. Cancer 1986;57:808–811.
17. Kallioniemi O-P, Banco G, Alavaikko M, et al.: Tumor DNA ploidy as an independent prognostic factor in breast cancer. Br J Cancer 1987;56:637–642.
18. Fallenius AG, Auer GU, Carstensen JM: Prognostic significance of DNA measurements in 409 consecutive breast cancer patients. Cancer 1988;62:331–341.
19. Beerman H, Kluin PM, Van de Velde CJH, Cornelisse CJ: Prognostic significance of DNA ploidy in a series of 690 breast cancer patients. Int J Cancer 1990;45:34–39.
20. Clark GM, Dressler LG, Owens MA, et al.: Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. N Engl J Med 1989;320:627–633.
21. Cornelisse CJ, van de Velde CJH, Caspers RJC, et al.: DNA ploidy and survival in breast cancer patients. Cytometry 1987;8: 225–234.
22. Del Bino G, Silvestrini R, Zucconi MR, et al.: DNA ploidy of human breast cancer. Anal Cell Pathol 1989;1:215–223.
23. Dressler LG, Seamer LC, Owens MA, et al.: DNA flow cytometry and prognostic factors in 1331 frozen breast cancer specimens. Cancer 1988;61:420–427.
24. Hedley DW, Rugg CA, Gelber RD: Association of DNA index and s-phase fraction with prognosis of nodes positive early breast cancer. Cancer Res 1987;47:4729–4735.
25. Jakobsen A, Poulsom HS, Madsen EL, et al.: Ploidy level of human breast carcinoma: Relation to histopathologic features and hormone receptor content. Acta Radiol Oncol 1984;23:103–107.
26. Raber MN, Barlogie B, Latreille J, et al.: Ploidy, proliferative activity and estrogen receptor content in human breast cancer. Cytometry 1982;3:36–42.
27. Frierson HF: Grade and flow cytometric analysis of ploidy for infiltrating ductal carcinomas. Hum Pathol 1993;24:24–29.
28. Bauer TW, Tubbs RR, Edinger MG, Suit PF, et al.: A prospective comparison of DNA quantitation by image and flow cytometry. Am J Clin Pathol 1990;93:322–326.
29. Clemo FA, Crabtree WN, Walker E, DeNicola DB: Comparison of image analysis and flow cytometric measurements of DNA content of canine transitional cell carcinomas. Anal Quant Cytol 1993;15:418–426.
30. Bloom HJG, Richardson WW: Histological grading and prognosis in breast cancer. Br J Cancer 1957;11:359–377.
31. Bacus JW, Bacus JV: A method of correcting DNA ploidy measurements in tissue sections. Mod Pathol 1994;7:652–664.
32. Sapi Z, Hendricks JB, Pharis PG, Wilkinson EJ: Tissue section image analysis of breast neoplasms. Evidence of false aneuploidy. Am J Clin Pathol 1993;99:714–720.
33. Snedecor GW, Cochran WG: ''Statistical Methods'' 7th Ed. Ames, IA: Iowa State University Press, 1980, p192.
34. Armitage P: ''Statistical Methods in Medical Research.'' New York: Blackwell Scientific, 1971, p127.
35. Harris JR, Hellsman S, Canellos GP, Fisher B: Cancer of the breast. In DeVita VT, Hellsman S, Rosenberg SA (eds): ''Cancer Principles and Practice of Oncology.'' 4th Ed. Philadelphia: JB Lippincott, 1993, p1310–1311.
36. Whitliff JL: Steroid hormone receptors in breast cancer. Cancer 1984;53:630–643.
37. Collan Y, Klemi P, Kallioniemi OP, et al.: Significance of variation in DNA flow cytometric analysis from paraffin embedded breast cancers. Pathol Res Pract 1992;188:581–586.
38. Kallioniemi OP, Joensuu H, Klemi P, Koivula T: Inter-laboratory comparison of DNA flow cytometric results from paraffin-embedded breast carcinoma. Breast Cancer Res Treat 1990;17: 59–61.
39. Askensten UG, van Rosen AK, Nilsson RS, Auer GU: Intratumoral variations in DNA distribution patterns in mammary adenocarcinomas. Cytometry 1989;10:326–333.
40. Ferno M, Baldetorp B, Ewers SB, et al.: One or more samplings for flow cytometric DNA analysis in breast cancer—Prognostic implications? Cytometry 1992;13:241–249.
41. Schutte B, Reynders MMJ, Bosman FT, Blijham GH: Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin-embedded tissue. Cytometry 1985;6:26–30.
42. Feichter GE, Goerttler K: Pitfalls in the preparation of nuclear suspensions from paraffin embedded tissue for flow cytometry. Cytometry 1986;7:616.
43. Stephenson RA, Gay H, Fair WR, Melamed MR: Effect of section thickness on quality of flow cytometric DNA content determinations in paraffin-embedded tissues. Cytometry 1986;7:41–44.
44. Frierson HF: Flow cytometric analysis of ploidy in solid neoplasms. Comparison of fresh tissues with formalin fixed paraffin-embedded specimens. Hum Pathol 1988;19:290–294.
45. Yung WK, Shapiro JR, Shapiro WR: Heterogeneous chemosensitivities of subpopulations of human glioma cells in culture. Cancer Res 1982;42:992–998.
46. Leith JT, Dexter DL, DeWyngaert JK, et al.: Differential responses to x-irradiation of subpopulations of two heterogeneous human carcinomas in vitro. Cancer Res 1982;42:2556–2561.
47. Manni A, Lancaster S, English H, et al.: Kinetic and morphometric responses of heterogeneous populations of NMU-induced rat mammary tumor cells to hormone and antipolyamine therapy in vivo. Breast Cancer Res Treat 1991;17:179–186.
48. Wersto RP, Liblit RL, Deitch D, Koss LG: Variability in DNA measurements in multiple tumor samples of human colonic carcinoma. Cancer 1991;67:106–115.
49. Dangou J-M, Kiss R, DePrez C, et al.: Heterogeneity of DNA ploidy, proliferation indices and nuclear size in human colorectal carcinomas. Anal Quant Cytol Histol 1993;15:23–31.
50. Stipa S, Danesi DT, Modini C, et al.: The importance of heterogeneity and of multiple site sampling in prospective determination of deoxyribonucleic acid flow cytometry. Surg Gynecol Obstet 1993;176:427–434.

## Commentary

More than 40 prognostic indicators have been documented for breast carcinoma, but while efficacy is significant by statistical testing, levels of positive predictive value generally are poor. No effective predictor is available for breast carcinomas less than 1 cm in diameter. Probability of relapse within seven years is only approximately than 10% in this group (1). With such a low risk, general application of adjuvant therapy is not justifiable. Probability of relapse exceeds 90% when ten or more lymph nodes contain metastases, but one would still welcome a means of identifying the minority not so destined. In the mid portion of the spectrum, addition of new prognostic markers to the standard tumor grade and stage measurements can enhance predictive accuracy, but still with no better than 90% accuracy when the prediction is against relapse within five years or 25 to 60% when the prediction is for relapse. While search for better markers should continue, we also appropriately should look at reasons for the imperfect prediction of those markers we

currently have. Regional heterogeneity of breast carcinoma is one such explanation.

The report by Dodd et al in this issue confirms prior studies of breast carcinoma heterogeneity and contributes new knowledge. Their finding of dramatic regional differences in Ki-67 protein expression is noteworthy because of utility of this measurement of proliferation in clinical oncology. We have observed similar sometimes dramatic regional differences in proliferation of breast carcinoma by in vitro 5′bromodeoxyuridine (BrdU) labeling (2). Potential explanations for regional proliferative differences can include: 1. Short-term changes in perfusion resulting from vascular constriction or dilatation. 2. Long-term differences in perfusion resulting from differences in capillary density from one region to another or dependent on adequacy of major vascular supply. 3. True heterogeneity of the neoplastic cell population wherein different cell lines occupying different sectors of the tumor differ in proliferative rates. The first explanation could readily account for differences in mitotic index (MI) or S-phase fraction (SPF) when the latter is measured by DNA precursor (tritiated thymidine, BrdU) incorporation. Ki-67 expression is not likely to respond rapidly to environmental effects. The half-life of Ki-67 protein has been measured as near one hour (3). After cells have ceased proliferating (entered $G_0$), the protein disappears, but its disappearance may require considerable time (4). Under conditions of deprivation slowly proliferating cells may retain Ki-67 protein although mitoses and S-phase cells are sufficiently rare to indicate a noncycling status by these criteria (3). Ki-67 protein remains detectable in MCF-7 cell lines arrested by tamoxifen (5). These findings suggest that a sharp cutoff of Ki-67 protein synthesis may occur when cells commit to postmitotic differentiation, but not when cycling neoplastic cells cease proliferation because of deprivation of oxygen or other environmental requirements. Regional environmental differences can not yet be dismissed as cause for proliferative heterogeneity observed, but problems with that explanation lead to consideration of the alternative suggestion that differences in proliferation may result from existence of inherently different cell lines in tumors.

Regional heterogeneity in breast carcinomas has implications on sampling procedures for studies of prognostic markers. Rational, practical sampling procedures are needed, and analysis of data will need to be modified appropriately. Sampling should include metastatic axillary lymph nodes when present, for the metastasizing cell lines are of greatest interest. Rather than the mean value obtained from enumerative data such as microscopic counts of proliferating cells, the standard deviation or other measures of dispersion should also be evaluated.

## References

1. Silverstein MJ, Gierson ED, Waisman JR, Senofsky GM, Colburn WJ, Bamagami P. Axillary lymph node dissection for T1a breast carcinoma. Is it indicated? Cancer 1994;73:664–7.
2. Meyer JS, Wittliff JL. Regional heterogeneity of breast carcinomas: thymidine labelling index, steroid hormone receptors, DNA ploidy. Int J Cancer 1991;47:213–20.
3. Bruno S, Darzynkiewicz Z. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif 1992;25:31–40.
4. Baisch H, Gerdes J. Simultaneous staining of exponentially growing versus plateau phase cells with the proliferation-associated antibody Ki-67 and propidium iodide: analysis by flow cytometry. Cell Tissue Kinetics 1987;20:387–91.
5. Duchrow M, Gerdes J, Schlueter C. The proliferation-associated Ki-67 protein: definition in molecular terms. Cell Prolif 1994;27: 235–42.

**John S. Meyer, M.D.**
St. Luke’s Hospital
Chesterfield, MO

Department of Pathology
Washington University School of Medicine
St. Louis, MO